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Spongy tissue development in Alphonso mango: association with *Staphylococcus xylosus*

Machhindra T. Janave · Arun Sharma

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Abstract A Gram-positive and catalase positive *Staphylococcus* strain was found to be associated with spongy tissue defect of Alphonso mango. The organism was identified to species level by physiological, morphological and biochemical characterization and fatty acid profile. The strain was identified as *Staphylococcus xylosus* (IMTECH, India, Accession No. MTCC 7441). The optimal growth of the organism was observed in the pH range of 5.0–9.0 and temperature range of 10–45°C. It was mannitol and arabinose-positive and able to produce acid from various sugars. The organism was able to grow in a medium containing 2–10% NaCl. It was further identified to species level by genomic sequencing of 1,387 base pairs of DNA (Gene Bank accession No. EU019195). Based on nucleotide homology and phylogenetic analysis, the microbe was found to be *S. xylosus*. The survey of Alphonso mango trees with a known history of producing spongy fruits showed that some of the twigs, leaves and flowers were coated with a honeydew-like sticky substance and mango hopper insects were observed over the inflorescence. The source of contamination of spongy fruits by *S. xylosus* was in the sticky mass. Gram staining, the catalase test and morphological features of the culture isolated from the sticky mass exhibited characteristics identical to the *Staphylococcus* strain isolated from the spongy pulp. Pathogenicity tests on different varieties of mangoes, apples and guavas indicated that the disease was experimentally transmitted from infected to healthy fruits. This observation suggested that this organism develops spongy symptoms in the fruits post-harvest and lacks specificity. In inoculated fruits, catalase and peroxidase enzymes were expressed as in naturally infected fruits. This report shows that the infection by *S. xylosus* could be a major initiating factor for spongy tissue development in Alphonso mangoes.

Keywords Internal physiological disorder · *Mangifera indica* · Spongy tissue · *Staphylococcus* · X-ray imaging

Abbreviations

BHI	brain heart infusion
BPA	Baird Parker agar
NA	nutrient agar
PCA	plate count agar
PDA	potato dextrose agar
PMSF	phenylmethanesulfonyl fluoride
POX	peroxidase

M. T. Janave (✉) · A. Sharma
Food Technology Division,
Bhabha Atomic Research Centre,
FIPLY, Trombay,
Mumbai 400 085, India
e-mail: mtjanave@barc.gov.in
e-mail: mtjanave@yahoo.com

Present address:
M. T. Janave
Laxmi Tower, B-101, Sector 42, Plot No. 7,
Opp, Seawoods Darave Rly. Stn., Nerul (W),
Navi Mumbai 400 706, India

PVPP polyvenylpolypyrrolidone
S. *Staphylococcus*
TSA tryptic soy agar

Introduction

Mango, *Mangifera indica*, grown throughout the tropics and subtropics, is one of the important fruit crops in the world. India produces over 70% of the world's mango and is a major exporter of this fruit. Alphonso, the most delicious variety of Indian mangoes famous for its excellent aroma and taste, accounts for about 60% of the mango export trade. However, this fruit is susceptible to an internal physiological disorder known as 'spongy tissue'. About 30% of the produce is affected by this disorder and is unfit for human consumption due to its poor quality and unacceptable off flavour. The diseased fruits do not show any external symptoms and the defect is detected only after cutting, posing a challenge for quality control. In diseased fruits, the pulp closer to the seed remains unripe and whitish in colour. The spongy pulp changes colour from faint yellow to brownish black, with or without air pockets accompanied with unacceptable off flavour. The occurrence of spongy defect is prevalent in the coastal Konkan region of Maharashtra State in India, the natural habitat of the Alphonso variety. Spongy tissue-affected fruits can be detected by the non-destructive X-ray imaging technology (Thomas et al. 1993).

The development of spongy tissue disorder has also been reported from other parts of the country, as well as in other mango-growing regions worldwide. Other varieties in India, namely, Goamankurd, Jamadar, Vanraj, Olour, Vellaikolamben, Swarnrekha and Fernandin grown in southern parts of India also show the presence of this disorder (Katrodia 1988). Our observations indicate that this disorder is also prevalent in the Balsad variety of Alphonso mangoes grown in Gujarat state, India (Janave 2006a). The occurrence of spongy tissue has been reported in Tommy Atkins mangoes grown in Brazil (Lima et al. 1999, 2001), Ching Hwang mangoes grown in Taiwan (Lee et al. 2000), cv. Carbao grown in Philippines (Dasuki 1987), mangoes grown in Malaysia (Lim and Khoo 1985) and in Florida, USA (Kader 2006; Paull 2002; Raymond et al. 1998a), and varieties Zill, Sensation and Tommy Atkins grown in South Africa (Oosthuyse 1997). The disorders of mango, internal breakdown (spongy tissue), jelly seed and soft nose are thought to be one and the same (van Lelyveld and Smith 1979; Lim and Khoo 1985). Soft nose disorder does not develop after harvest unless it was present at harvest (Young and Miner 1961). In general, about 30% or in extreme cases about 60% of the produce (Vasanthaiah et al. 2006) is affected by this disorder. Also, this disorder has tremendous adverse impact on the export potential of the famous Alphonso variety grown in India.

Several investigations have been carried out in the past to understand the causes of spongy defect, but without success. Spongy tissue formation has been associated with factors as diverse as nutritional, ecological, physiological and biochemical (Gupta et al. 1985; Katrodia 1988; Lima et al. 2001; Raymond et al. 1998b). Some of the probable causative factors thought to be responsible include deficiency of nutrients such as calcium (Gunjate et al. 1979; Raymond et al. 1998a), bacterial infection (Chhatpar et al. 1968–1969), late harvesting or the fruits ripened on the tree (Katrodia 1988), high temperature (Katrodia and Rane 1989; Ravindra and Shivashankar 2004), excessive tree vigour (Katrodia and Seth 1989) and lower fruit transpiration (Shivashankar and Mathai 1999). Attempts have been made in the past to control this disorder, but no foolproof solution has yet been achieved. Pre-harvest Ca sprays or post-harvest Ca solution dip treatments have been employed to control spongy tissue (Raymond et al. 1998a; Oosthuyse 1997). However, the literature shows contradictory findings; in some cases increased incidence of spongy tissue was observed after calcium sprays (Oosthuyse 1997). The findings by Raymond et al. (1998a) indicated that internal breakdown could not be specifically linked to Ca deficiency at any stage of fruit ontogeny. Storage of the fruits at ambient temperature along with good aeration to prevent excessive heat and to avoid O_2 has been employed to reduce the extent of this defect (Dasuki 1987). Harvesting fruits at 80% maturity has been adopted in certain mango-growing regions in India to avoid spongy tissue. A combination of FYM and Micnelf spray is found to result in a significant decrease in spongy tissue incidence by the group at the Central Experimental Station, Institute of Micronutrient Technology, Konkan Agricultural University, Dapoli, Maharashtra, India, (www.ranadey.com/comm.htm). Also, pre-harvest treatment with paclobutrazol resulted

in a considerable reduction of spongy tissue incidence (Ravindra and Shivashankar 2004). Despite all these treatments, no effective method is available to eliminate spongy tissue completely, probably due to the fact that the actual causative agent of spongy tissue formation is not yet known. A recent study indicated that spongy tissue development was closely associated with the shift of the seed into germination mode (Ravindra and Shivashankar 2004). The differentially expressed genes related to various enzymes specific to spongy tissue have been cloned (Vasanthaiah et al. 2006). These studies also are largely focused on the biochemical and enzymological changes in the pulp and seed.

Despite all the biochemical and enzymological studies, the actual agent responsible for this disorder has not been identified. No report is yet available to correlate spongy tissue development with microbial infection, except by Chhatpar et al. (1968–1969). In a laboratory investigation when the diseased pulp was plated on plate count agar (PCA) plates, bacterial colonies appeared (Janave 2006a). The observation was repeated several times observing all the aseptic techniques. This observation indicated an association of a bacterium with the diseased pulp. As a result of this bacterial infection, several biochemical changes such as increased respiration rate, loss of carotenoids associated with lipoxygenase-mediated β-carotene co-oxidation, increase in peroxidase (POX) activity, decrease in soluble protein and increase in total phenolic content were observed (Janave 2007). The present investigation reports further studies on the isolation, identification and characterization of a bacterium from the diseased fruits and its association with spongy tissue disorder. The characteristics of the bacterium isolated from the spongy pulp and the results of the pathogenicity tests on various fruits are reported. Attempts have been made to correlate high levels of catalase activity in the bacterial protein, an increased rate of respiration and POX activities in the spongy fruits with the air pocket formation and the blackening of the pulp.

Materials and methods

Plant material

Mango fruits cv. Alphonso from the coastal Konkan region of Maharashtra state during harvesting seasons 2005–2007 were obtained from a local market. The fruits were scanned for spongy tissue presence under an X-ray imaging machine (ECIL XBS 9050S, Electronic Corporation of India Limited, Hyderabad, India). During the 2006 season, Alphonso trees at Bhabha Atomic Research Centre (BARC) campus were surveyed at the time of flowering to determine if the trees could be the probable target of spongy tissue development in the fruits. Some of the twigs and leaves were found coated with a sticky mass, and the presence of mango hopper insects was observed over the inflorescence. The affected branches were removed, separated into sticky leaves and flowers and used for the isolation of bacteria.

Microbiological studies

Healthy and spongy fruits selected through X-ray imaging were washed thoroughly under tap water, allowed to dry at room temperature and transferred to a laminar flow bench. The fruits were surface-sterilized by rubbing with 70% ethanol-soaked sterile cotton and the ethanol allowed to evaporate dry. The fruits were then cut open aseptically. The white corky spongy tissue from the diseased fruits and healthy tissue from the non-spongy fruits were transferred to 90 ml sterile brain heart infusion (BHI, HIMEDIA) broth and homogenized in a sterile Omni mixer cup. One millilitre of homogenate after serial dilution was pour-plated on potato dextrose agar (PDA), PCA and Baird Parker agar (BPA) plates. PDA plates (pH 3.5) were prepared containing sterile tartaric acid to arrest bacterial growth and support growth of yeasts and moulds. PDA plates were incubated at room temperature (28–30°C) and PCA and BPA plates at 37°C. The total bacterial load was determined per gram of pulp. Well-separated pure colonies from PCA and BPA plates were selected for further sub-culturing by repeatedly streaking on fresh PCA or BPA plates. The isolated colonies were tested for Gram reaction and viewed under a Carl Zeiss (Model Axiolab Ere, Germany) microscope fitted with a camera (Axiocam MRc). Catalase test was conducted by putting a drop of 3% H_2O_2 on the colonies.

The microbiological studies were carried out on fruits obtained during three seasons (April–July) in the years 2005, 2006 and 2007. During each harvesting season, minimum of 50–75 fruits (each of non-spongy and spongy) were obtained three times and analyzed for spongy tissue detection by passing

through the X-ray imaging machine and for the isolation of bacteria. For bacterial isolation, about ten fruits were cut open and four well-developed spongy fruits were selected. Only spongy pulp adhering to the seed was removed and used for bacterial isolation as detailed above. Similarly, healthy pulp from the non-spongy fruits was tested for the presence of the bacteria. The other parts of the mango tree producing spongy tissue-affected fruits (leaves and inflorescence), were also tested for the presence of the microorganism. The bacteria were isolated from sticky leaves and flowers as detailed above and were selected for further sub-culturing, Gram staining and the catalase test.

Preliminary characterization of the isolates

Isolates were plated out on different media to determine appropriate media for supporting growth. Total microbial load was determined on PCA and BPA plates after serial dilution and pour-plating. The isolates were biochemically characterized on different sugars for their utilization. Identification of the organism to genus level was done in the laboratory on the basis of colony morphology, Gram staining and the catalase test. The culture was sent for identification to species level to the Institute of Microbial Technology (IMTECH), Chandigarh, India, for morphological, physical and biochemical characterization and to Bangalore Genei, Bangalore, India for genomic DNA sequencing and phylogenetic analysis. Fatty acid analysis was done through the services of the Commonwealth Agriculture Bureaux International (CABI) Bioscience, UK.

Isolation of genomic DNA, sequencing and phylogenetic analysis

Genomic DNA was isolated from pure culture pellets using the Genei Ultrapure Genomic DNA prep kit for bacteria (KT 83B). The consensus primers were used to amplify the ~1.5 kb 16S ribosomal DNA (rDNA) fragment using high-fidelity polymerase chain reaction (PCR) Polymerase (Taq DNA polymerase). For cloning, pGEM-T Easy vector from Promega was used for the PCR amplified product to be cloned in the vector. For transformation, *Escherichia coli* DH5 α was used and then plated on Luria–Bertani (LB) agar containing ampicillin (20 μl of 100 mg ml^{-1}), X-gal (25 μl of 50 mg ml^{-1}) and isopropyl β-D-1-thiogalactopyranoside (40 μl of 0.1 M). The colonies were inoculated in LB broth and the screening of 24 colonies yielded three clones of which two were sequenced. The PCR product was cloned and plasmid DNA was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data were aligned and analyzed for finding the closest homologue for the microbe, and the phylogenetic tree was constructed based on 16S rDNA sequence data. The nucleotide sequence was aligned using Clustal X and Gendoc programmes. The construction of the phylogenetic tree was performed by the neighbour-joining method and evaluated by bootstrap analysis. The analysis was carried out by Bangalore Genei, Bangalore, India.

Pathogenicity testing

The pathogenicity of the bacterial isolate was ascertained using Koch's Postulates. The pure organism was grown in 100 ml BHI overnight and centrifuged at 10,000×*g* for 10 min. The pellet was dissolved in 1.5 ml sterile saline and 0.1 ml aliquot (1×10^7 cfu ml^{-1}) was used for inoculation of the healthy mango fruits. Different varieties namely, Alphonso, Kesar, Rajapuri, Totapuri and Badami were passed through the X-ray imaging machine to select only non-spongy fruits. Five surface-disinfested fruits from each variety were inoculated by two methods: (1) slicing the ripe fruits and inoculating and (2) puncturing the unripe fruits and inoculating. An incision was made with the help of a sterile stainless steel tube (2 mm ID) and 0.1 ml of the culture was transferred into the cavity under aseptic conditions. The cavity was closed with the cut portion of the fruit. The fruits without and with incision, but not inoculated, served as the control. The fruits were stored in 3 l beakers covered by double-layered muslin cloth. A beaker containing sterile distilled water was kept beside the fruits to avoid moisture loss due to desiccation. Fruits were observed for symptoms of spoilage and cut open after 8–9 days. The spoiled pulp was used for the extraction of bacteria in BHI medium and the homogenate was plated on PCA and BPA plates to confirm that the same organism could be recovered from inoculated fruits.

Biochemical studies

Healthy and damaged fruits were cut open at various stages of ripening. Pulp samples were collected from the respective fruits and used immediately or stored at −20°C after freezing with liquid N_2. The pulp samples were used for the extraction of POX and catalase activities (Janave 2006b). The spoiled pulp (10 g) from inoculated fruits and fruits at various stages of ripening was transferred to a pre-chilled pestle and mortar and powdered to a fine paste by using liquid N_2. The paste was extracted with 50 ml of 0.05 M potassium phosphate buffer pH 6.5 (buffer A) containing 2% polyvenylpolypyrrolidone, 0.05% ascorbic acid, 0.01% phenylmethanesulfonyl fluoride, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 2 mM ethylenediaminetetraacetic acid (EDTA), and centrifuged at 27,000×*g* for 15 min at 4°C. To the supernatant, three volumes of chilled acetone was added and again centrifuged. The precipitated proteins were dissolved in buffer A and the protein content was determined by the Bradford dye-binding method (Bradford 1976).

For the assay of catalase and POX activity in the culture, the cells were grown in BHI medium overnight and centrifuged at 10,000×*g* for 10 min at 4°C. The pellet was washed with 10 ml of buffer A and centrifuged again as before. The cells were then suspended in 10 ml buffer A, containing 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EDTA and 10% glycerol and sonicated for 30 s cycles for 2 min and centrifuged as previously described.

Catalase and POX assay

The catalase activity was determined by the change in absorbance of H_2O_2 at 240 nm (Beers and Sizer 1952). One millilitre reaction mixture contained 0.05 M buffer A, 0.03% H_2O_2 and an enzyme fraction. The reaction was followed in time and the enzyme activity was expressed as ΔA_{240}nm min^{-1} mg^{-1} protein. The POX activity was determined by the change in absorbance at 460 nm due to the oxidation of *o*-dianisidine. One millilitre reaction mixture contained 0.05 M citrate phosphate buffer, pH 5.0, 0.03% H_2O_2, 0.25 mM *o*-dianisidine and an enzyme fraction. The rate of reaction was followed in time and the activity was expressed as ΔA_{460}nm min^{-1} mg^{-1} protein.

Results and discussion

Spongy tissue characteristics

No external symptoms were observed in the spongy tissue-affected fruits (Fig. 1a). Therefore, the X-ray

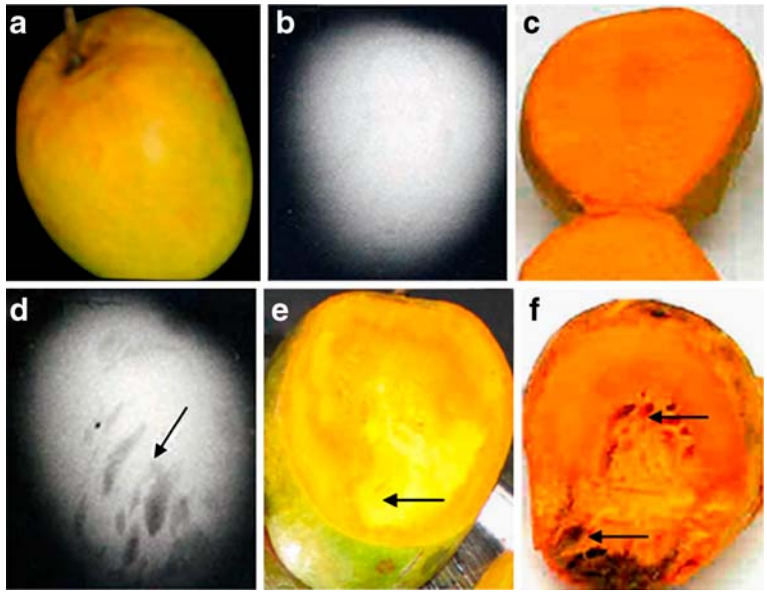

Fig. 1 Spongy tissue characteristics of Alphonso mango fruits: **a** spongy whole fruit; **b** X-ray image of non-spongy fruit; **c** non-spongy cut fruit; **d** X-ray image of spongy fruit, the *arrow* indicates white patches due to air cavity in spongy fruit; **e** spongy cut fruit, the *arrow* indicates spongy-white pulp adhering to the seed and **f** spongy fruit with air cavities, the *arrow* indicates air cavities and pulp turning brown at spongy-black stage

imaging machine was used for the identification of spongy defect by passing the whole fruits under the scanner prior to cutting. The X-ray image of the healthy fruits showed normal homogenous pulp without any internal patches (Fig. 1b) and the fruit was confirmed to be non-spongy after cutting (Fig. 1c). However, the affected fruits displayed white patches in the internal flesh in the X-ray scanner (Fig. 1d, indicated by an arrow) and were found to be spongy after cutting (Fig. 1e). White unripe pulp affected by the spongy defect was found firmly adhering to the seed (Fig. 1e, indicated by an arrow) surrounded by the ripe brown pulp. As ripening progressed, the white pulp turned soft and then became yellowish to brownish black with air cavities (Fig. 1f). The typical X-ray image of spongy fruits was due to the density difference caused by uneven ripening and cavity formation (Thomas et al. 1993). About 150–200 fruits were scanned by X-ray imaging and each time about 30% fruits were detected as spongy. Almost a 90% correlation was observed in the fruits detected as spongy by X-ray scanning and then cutting (Janave 2007).

The development of spongy tissue was accompanied with characteristic biochemical changes induced by this defect (Janave 2007). The adherence of whitish pulp to the seed coat in spongy fruits could be due to the poor pectin-degrading activity (Chitarra et al. 1999) and the inability to synthesize ripening-associated carotenoids (Janave 2007). The development of a brownish–black colour could be caused by increased POX activity. About a twofold higher respiration rate was observed in the spongy fruits throughout the storage period compared to that in non-spongy fruits (Janave 2007). The cavities observed in spongy fruits are reported to be caused by increased respiratory activity (Ravindra and Shivashankar 2004). Our studies indicated a decrease in catalase activity in spongy-white pulp, and in the spongy-black pulp the activity was restored showing almost identical levels as in non-spongy fruits (Janave 2007). Vasanthaiah et al. (2006) have shown higher expression of catalase gene in spongy tissue-affected fruits. Other reports on biochemical studies also show many compositional and metabolic differences between the healthy and damaged tissues (Gupta et al. 1985; Katrodia 1988; Lima et al. 2001; Vasanthaiah et al. 2006).

Microbiological studies

Identification and characterization of the isolate The spongy pulp from Alphonso fruits when plated on to PCA plates displayed the presence of bacterial colonies. Creamy-white colonies appeared after incubation at 37°C for 24 h (Table 1). On BPA plates, black colonies appeared within 24–48 h. Only a single type of colony morphology was observed on PCA and BPA plates indicating the presence of a single type of bacterium. When the isolate was cultured on other media plates like PDA, Simmons citrate agar and McConkey agar, no growth was observed (Table 1). The growth of the isolate on BHI agar and tryptic soy agar media resulted in yellowish colonies, whereas nutrient agar produced creamy-white colonies as on PCA plates. The isolation of the organism from fruits obtained in different harvest seasons confirmed the presence of the same organism in spongy fruits. In the 2005 harvesting season, the isolation of the organism was carried out three times from April to June, and three cultures were obtained. All three cultures exhibited identical characteristics as above. At all times only a single type of bacterial colony was observed. On PDA plates, no growth was observed even after 7 days, indicating the absence of yeasts or moulds. Non-spongy fruits did not show any growth even after prolonged incubation for up to 7 days.

The cocci isolated from spongy pulp when cultured on PCA plates produced colonies that were circular in shape, smooth, raised and glistening. The cells occurred predominantly in pairs or as grape-like clusters, characteristic of *Staphylococcus*. The organ-

Table 1 Growth of isolated *S. xylosus* from spongy Alphonso pulp on different media

Media	Growth	Colour
Plate count agar	+	Creamy-white
Brain heart infusion agar	+	Yellowish
Nutrient agar	+	Creamy-white
Tryptic soy agar	+	Yellowish
Baird Parker agar	+	Black
Potato dextrose agar	−	–
Simons citrate agar	−	–
McConkey agar	−	–

+ Positive growth, − negative growth

ism was found to be catalase-positive and Gram-positive. In spongy-white fruits upon serial dilution on PCA plates, the bacterial load was found to be around 1×10^5 cfu g^{-1} pulp. On BPA plates the organism developed circular black colonies typical of *Staphylococcus* and the bacterial load was found to be around 5×10^4 cfu g^{-1} pulp.

The morphological, physiological and biochemical characteristics of the organism observed by IMTECH are presented in Tables 2 and 3. The creamy colonies were entire, opaque, non-endospore forming, and non-motile with a diameter of 0.5–0.8 μm (Table 2). The motility test was carried out by IMTECH using the drop method. The pure culture showed growth at temperatures of between 10°C and 45°C and at a pH in the range of 5–9. Optimum growth was obtained at 30°C and at pH 5. The organism was able to grow in a medium containing 2–10% NaCl. The organism was mannitol and arabinose-positive and able to produce acid from various sugars (Table 3). The pure culture showed negative oxidase, lipase, citrate utilization and starch hydrolysis tests. Based on these biochemical and morphological characteristics, the organism was identified as *Staphylococcus xylosus* (MTCC 7441). The fatty acid profile obtained by CABI Bioscience (IMI 393389) contained saturated fatty acids (Table 4). The fatty acid profile clearly identified the bacterial isolate as a member of the genus *Staphylococcus*. The fatty acid compositions of the coagulase-negative *Staphylococci* are very similar, differing quantitatively, but not qualitatively (Wieser and Busse 2000; Kotilainen et al. 1991).

Table 2 Morphological and biological tests of isolated *S. xylosus* from spongy Alphonso pulp

Tests	Results
Configuration	Circular
Margin	Entire
Elevation	Raised
Surface	Smooth, glistening
Pigment	Cream
Opacity	Opaque
Gram reaction	+
Cell shape	Cocci
Size	0.5–0.8 μm
Arrangements	Clusters
Endospore	–
Motility	–
Fluorescence	–
Growth temp	10–45°C
Growth pH	5.0–9.0
Growth on NaCl	2.0–10%
Growth anaerobic	+

+ Positive reaction, − negative reaction.

Table 3 Biochemical characteristics of *S. xylosus* isolated from spongy Alphonso pulp

Tests	Results
Indole test	–
Methyl red test	+
Voges Proskauer test	+ (w)
Citrate utilization	–
Casein hydrolysis	+ (w)
Starch hydrolysis	–
Nitrate reduction	+
Catalase test	+
Oxidase test	–
Lipase test (Tween 80)	–
Acid production from:	
Arabinose	+
Cellobiose	+
Dextrose	+
Lactose	+
Maltose	+
D-Mannitol	+
Melibiose	+ (w)
Mannose	+
Rhamnose	+
Sucrose	+
i-Inositol	–
Inulin	–

+ Positive reaction, − negative reaction, + *w* positive to weak reaction

Identification by genomic DNA sequencing and phylogenetic analysis also identified the bacterium as *S. xylosus*. Based on the 1,387 base pairs nucleotide homology and phylogenetic analysis the microbe was identified as *S. xylosus* (GenBank Accession Number: EU019195). This strain showed 99% homology with DQ814743 and AF515587 identifying it as *S. xylosus* (Fig. 2). Nearest homologue species was found to be *Staphylococcus saprophytics* (Accession No. AM237352). Identification by the two methods, biochemical and morphological characterization, and DNA sequencing showed that the bacterium isolated from spongy Alphonso pulp belonged to the genus *Staphylococcus* and species *xylosus*.

Table 4 Fatty acid composition of *S. xylosus* strain isolated from spongy pulp of Alphonso mango

Fatty acid	Content (%)
$C_{14:0}$ iso	1.78
$C_{15:0}$ iso	9.71
$C_{15:0}$ anteiso	32.88
$C_{16:0}$ iso	1.8
$C_{16:0}$	0.95
$C_{17:0}$ iso	8.62
$C_{17:0}$ anteiso	10.8
$C_{18:0}$ iso	1.96
$C_{18:0}$	9.47
$C_{19:0}$ iso	6.46
$C_{19:0}$ anteiso	4.91
$C_{19:0}$	1.12
$C_{20:0}$	9.57

Characterization of bacterium isolated from the inflorescence To determine the source of infection, mango trees in the BARC complex with a known history of producing spongy fruits were surveyed during flowering. It was observed that some of the leaves and twigs were coated with a sticky mass (Fig. 3a) and were surrounded by Mango hopper insects in the vicinity of the inflorescence (Fig. 3b). The source of infection of this organism was found in the honeydew-like sticky mass on the leaves and flowers (Fig. 3a). The bacterial isolates from the sticky mass on leaves and flowers were characterized by Gram staining and colony morphology. Out of the three isolates, two were found to be Gram-positive cocci and the other was Gram-negative and rod-shaped. One of the Gram-positive isolates showed identical staining and morphological features to that isolated from the spongy pulp. This organism developed black colonies on BPA plates and creamy-white colonies on PCA plates. The pure culture was catalase-positive. These features were exactly identical with the bacterial culture isolated from the spongy pulp (MTCC 7441). The same bacterium was present in both the sticky mass before initiation of the fruit growth and in the spongy pulp of the fully mature fruit. Identification of the other two strains observed in the sticky mass is of further interest.

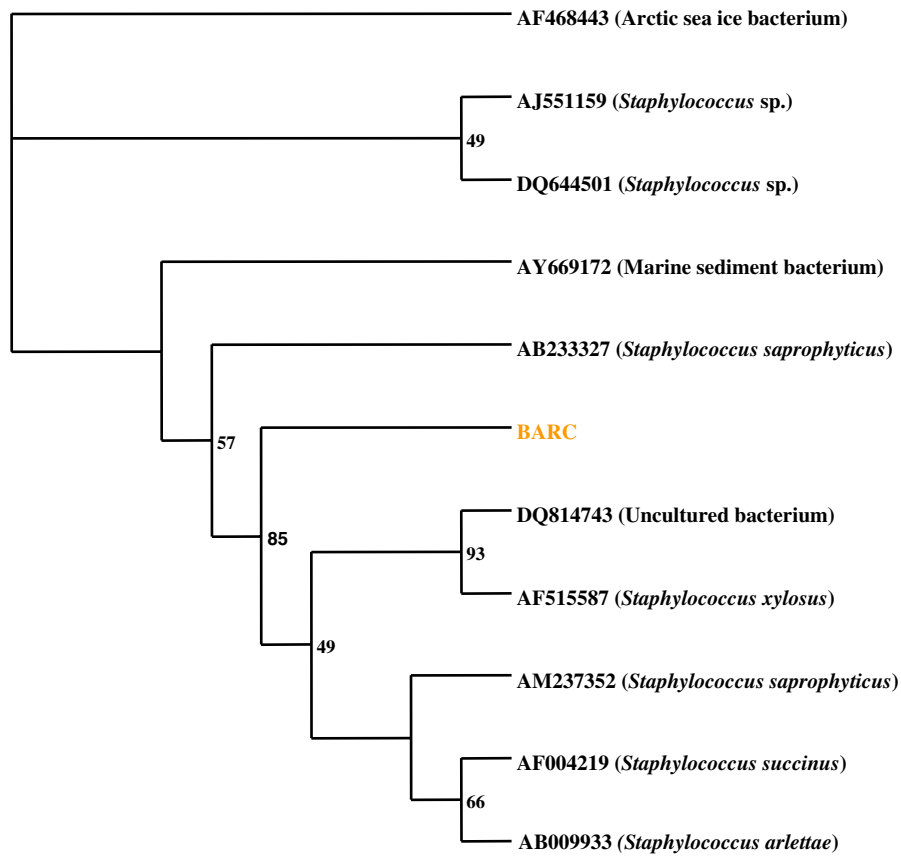

Fig. 2 Phylogenetic tree showing relationships among 11 species of the genus *Staphylococcus*. The tree was established from the analysis of the sequence of 1,387 base pairs of the bacterial strain isolated from spongy Alphonso fruit (shown as *BARC* in the tree) by using the neighbour-joining method. The numbers are the estimated confidence levels, expressed as percentages for positions of the branches determined by bootstrap analysis. The Accession number of the *Staphylococcus* strain from spongy Alphonso pulp (BARC) is EU019195

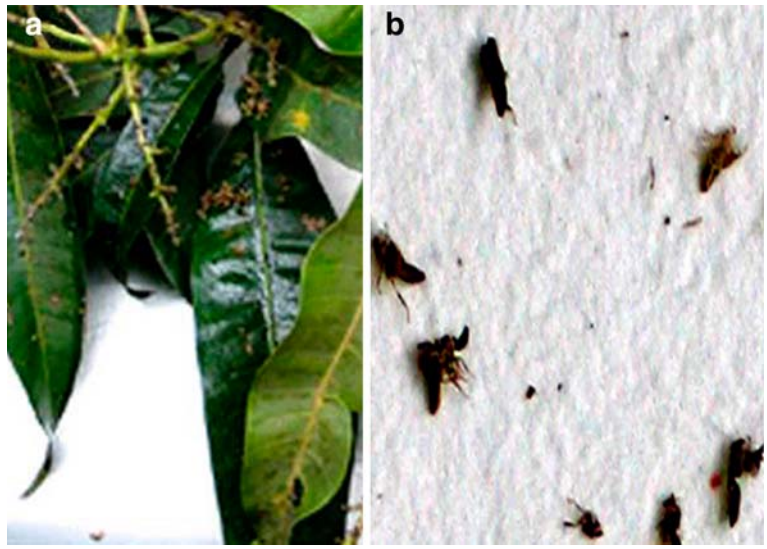

Fig. 3 **a** The sticky mass on the leaves and **b** mango hopper insects on the leaves and branches of Alphonso mango tree during flowering

The isolation of the same organism from the sticky mass on the inflorescence and the spongy pulp (MTCC 7441) suggests some possibilities about the mode of entry of this organism in the fruits. One possibility that needs to be confirmed could be that the mango hopper insects may harbour these bacteria. The mango hoppers generate sweet honeydew-like sticky masses by sucking the cell sap between the tender branches and the flowers (http://www.horticulture world.net, 2005). The other possibility could be that this organism may be air-borne. In the winter season of November–December, there is occasional fog, which could precipitate the microbes from the environment on the sticky masses generated by mango hoppers. Also, spongy tissue development is found to be more pronounced after early stormy rain, further suggesting air-borne infection. The presence of the *Staphylococci* strain in the sticky masses indicates that the microbe may possibly enter the fruits through the micro wounds created by the insects during the early stages of fruit development; after entry, it may remain dormant until the fruit matures. Once the seed epicoat is formed, the bacteria could attach and multiply in the pulp adhering to the seed (Fig. 1e). Other types of bacteria were also present in the sticky masses, but they were not found in the spongy pulp. It is not clear how only the *S. xylosus* strain isolated from the spongy pulp manages to enter the fruit and survive. How it survives the internal physiological environment of the growing fruit and multiplies, leading to spongy tissue development is not clear.

The role of insects as potential vectors of bacterial pathogens in fruits has been reported. Mediterranean fruit flies contaminated with *E. coli* were capable of transmitting this organism to apple (Sela et al. 2005). Also, different strains of *E. coli* have been isolated from apple maggot (Lauzon 2003) supporting the hypothesis that other fruit-foraging insect species have the potential to serve as vectors in the contamination of fresh produce (Sela et al. 2005). These observations lead to the conclusion that if the flowers are contaminated with the bacteria, the fruits are also likely to become contaminated. A recent study by Guo et al. (2001) indicates that when tomato plants were inoculated with *Salmonella* at the blooming stage, the pathogen was able to survive throughout the course of fruit development and maturation. The presence of epiphytal flora within tissues of fruits and vegetables through various pathways has been reported (Samish et al. 1963). By examining eight internal locations of tomatoes, they observed that bacteria are unevenly distributed in the fruit, and entry may be from the stem scar tissue through the core and into the endocarp. This study suggested that some epiphytal flora might reach the internal tissue of tomato through natural apertures because of their small size. It may be that bacteria enter fruit tissue more readily in the early stages of fruit development, at a time when various channels are yet to be covered

by corky or waxy materials (Samish et al. 1963). Further studies on inoculation of the isolated *S. xylosus* strain from the spongy pulp to the young mango fruits, and the monitoring of its survival throughout the fruit maturation will provide confirmative evidence for in vivo development of spongy tissue in mango fruits.

Mode of entry of bacteria into fruits and cross-pathogenicity As non-spongy fruits did not show any growth on PDA, PCA or BPA plates, the organism isolated from diseased fruits was tested for Koch's Postulates to confirm its association with spongy tissue development in Alphonso fruits. The pathogenicity testing showed that inoculation with the bacterial isolate induced spongy symptoms in mango fruits (Fig. 4). The control Alphonso fruit sliced or punctured mechanically without inoculum did not result in spoilage. The ripe fruits sliced and inoculated with the bacterial isolate developed spoilage within 3 days (Fig. 4b) with severe blackening of the pulp associated with tiny air pockets, as was the case in naturally infected spongy fruits (Fig. 1f). The slightly semi-ripe fruits inoculated with the isolate showed symptoms of typical spoilage due to spongy tissue formation (Fig. 4c). Spoilage occurred after 8–9 days, and the pulp surrounding the inoculum turned brownish–black with bigger cavities. Similar spoilage symptoms were observed after inoculation in Kesar, Badami and Rajapuri mangoes (Fig. 4e–g). In Totapuri mangoes, the spoilage was comparatively low (data not presented). Inoculation of the organism in other tropical fruits e.g. apples and guavas, also induced spoilage but without air cavity formation (Fig. 4i,j), except Cavendish bananas, which did not show any spoilage (data not presented). The control fruits, Alphonso and Kesar mangoes, or apples punctured mechanically and allowed to ripen under identical experimental conditions, along with the inoculated fruits did not result in spoilage (Fig. 4a,d, h) indicating that there was no external infection in the fruits during the experiment. Similarly, other control fruits Rajapuri, Badami and Totapuri or guava fruits also did not show any spoilage (data not presented). This also confirmed that the sterile water kept beside the fruits to control losses due to desiccation, and the muslin cloth used to cover beakers were not permeable to any other microbe from the environment. Post-harvest inoculation studies showed that all the fruits tested developed spoilage with air cavities indicating that this organism is not specific to the Alphonso variety and can also affect other fruits. The spoiled pulp from Alphonso mangoes after inoculation showed the presence of the same *Staphylococcus* strain as characterized by Gram staining, the catalase test and morphological features.

Spongy tissue has not been reported in any other mango except certain southern varieties, especially Alphonso in India. However, the pathogenicity tests showed that this defect could be induced in vitro in other mango varieties such as Rajapuri, Kesar, Badami or other fruits such as apples and guavas. It is intriguing to speculate on the biochemical factors that could lead to the susceptibility of Alphonso mangoes to *S. xylosus*. It is evident that Alphonso has some specific biochemical component that encourages the internal survival of contaminants and growth of this organism. One possible factor could be the typical aroma of Alphonso, that may attract the insects to the inflorescence and result in infection of fruits.

Biochemical studies

Catalase and POX activities in the cultured organism and fruits The intracellular protein extracted from the bacterial isolate showed high catalase activity (Table 5). Very low catalase activity was detected in the extracellular fraction of the bacterial culture. No POX activity was detected either intracellularly or extracellularly. In spongy-white pulp, POX activity was found to be twofold higher than that in ripe non-spongy fruits (Table 5). Slight reduction in catalase activity was observed in the spongy-white pulp. This decrease in catalase activity could be due to the affected pulp remaining unripe, as very low catalase activity was observed in unripe fruits (Janave 2007). Inoculated fruits showed increased levels of POX and catalase activities (Table 5).

Increase in POX activity in spongy pulp could explain the blackening of the pulp due to expression or activation of POX isoenzymes as the result of bacterial infection. Many peroxidases are expressed as a defence mechanism against pathogen attack (Hammerschmidt et al. 1982; Lagrimini et al. 1993). Increased rates of production and accumulation of

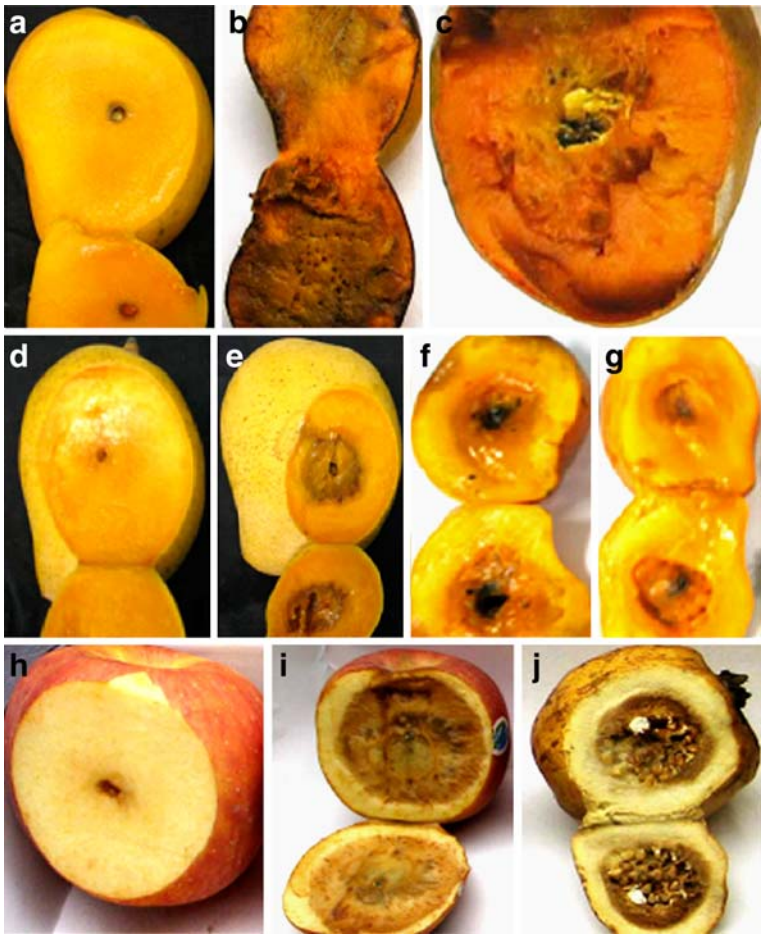

Fig. 4 Development of spoilage after inoculation of the strain in different fruits: **a** Alphonso punctured control; **b** Alphonso-sliced inoculated; **c** Alphonso punctured inoculated; **d** Kesar control; **e** Kesar inoculated; **f** Badami inoculated; **g** Rajapuri inoculated; **h** Apple control; **i** Apple inoculated; and **j** Guava inoculated fruits

reactive oxygen species (ROS) including H_2O_2, organic peroxide and superoxide, are important components of active plant defence responses to microbial invasion (Levine et al. 1994). For successful plant invasion, these ROS must be rapidly detoxified. Plant catalases and peroxidases are ubiquitous enzymes that are believed to play a vital role in a plant's defence mechanism against attack by pathogens. Vasanthaiah et al. (2006) also observed higher expression of catalase and concluded that oxidative stress may be one of the causes for the formation of spongy tissue. However, their studies do not mention the peroxidase gene, although our studies and those of Lima et al. (1999) indicate higher POX activity in spongy fruits. The generation of air cavities within 3 days after inoculation in the ripe fruits (Fig. 4b) and the increase in the size of the cavities during 8 days (Fig. 4c,e,g) suggests that these cavities

Table 5 Catalase and peroxidase activities at different stages of spongy tissue development in Alphonso mango and in the bacterial protein of the pure strain

Sample	Catalase ΔA_{240}nm min^{-1} mg^{-1}	Peroxidase ΔA_{460}nm min^{-1} mg^{-1}
Ripe-non-spongy	2.98±0.07	3.87±0.1
Spongy-white	1.8±0.2	7.96±0.12
Inoculated-spongy	2.05±0.05	6.06±0.06
Bacterial culture	0.90±0.04	–

The data is the average of triplicate readings for four experiments during 2005–2007 harvesting seasons ±SD.

could be due to the expression of high levels of catalase by the bacterial isolate. Although the data in Table 5 indicate that both the healthy and the spongy fruits showed almost identical catalase activity, in healthy fruits no cavities were observed. It is likely that the host fruits generated higher H_2O_2 levels for inactivation of the pathogen (Levine et al. 1994). Hence, in vivo, catalase activities after inoculation may be much higher than found in vitro. Further studies on catalase activity determination at different time intervals after inoculation may provide a satisfactory answer to the role of the catalase activity of *S. xylosus* in generating large amounts of O_2, resulting in pulp disruption and formation of air cavities. Generation of ROS including H_2O_2, or the expression of higher levels of POX isoenzymes could be the resistance response of the host against bacterial infection. Our parallel studies on POX indicated higher expression of anionic and cationic isoforms of POX in spongy fruits. Further studies on purification of these isoenzymes are underway to understand their precise role in the defence mechanism.

The presence of the *S. xylosus* strain in spongy pulp, inoculated fruits, sticky masses on leaves and flowers indicates that environmental infection during flowering is probably responsible for the entry of this organism in Alphonso fruits. This microbe may also gain entry into fruits by the micro wounds created by the mango hopper insects. The organism may remain dormant until the fruits mature, and start multiplication when the internal physiological conditions of the fruit, e.g. pH, are conducive for its growth.

The foregoing results suggest that *S. xylosus* is associated with the development of spongy tissue in Alphonso mango. *Staphylococcus* is an opportunistic organism and this pathogen has been isolated from a wide variety of fresh produce such as alfalfa sprouts, carrots, lettuce, onion sprouts and radish (Abdelnoor et al. 1983), and from rotting egusi fruit (Obeta and Ariba 1994). *Staphylococcus* spp. are also frequently isolated from the gut of Mexican fruit flies (Kuzina et al. 2001). However, no report is yet available to relate this infection with any kind of disease in fruits. As the development of spongy tissue is observed only when the fruits are harvested at full maturity (Katrodia 1988), the pH of the fruit may be an important factor for the development of spongy tissue to detectable levels. As the organism could not grow below pH 4.0, harvesting fruits below 80% maturity may explain why spongy tissue is not detected at this stage, as the pH will be around 3.0.

Some bacterial diseases of the mango tree caused by *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Erwinia carotovora* subsp. *carotovora* (Bradbury 1986), *Xanthomonas campestris* pv. *mangiferacindicae* (Ploetz et al. 1994; Cazorla et al. 1998) and *Pseudomonas syringae* have been reported (Gagnevin and Pruvost 2001). In apical necrosis, mango buds, leaves and stems are susceptible to infection but fruit lesions have not been observed. Bacterial black spot in many varieties resulted in drastic yield loss, associated with premature fruit drop (Gagnevin and Pruvost 2001). Leaf and fruit symptoms are most common and fruit susceptibility increases during the month preceding harvest. The fruit symptoms appear as small water-soaked spots exuding infectious gum on the surface. On the contrary, the spongy Alphonso fruits do not show any external symptoms of infection.

In conclusion, the results presented in this paper provide evidence for the association of spongy defect in Alphonso mango with a Gram-positive *Staphylococcus*. Biochemical characterization, genomic DNA sequencing and phylogenetic analysis identified the isolate as *S. xylosus*. Pathogenicity testing by Koch's Postulates reproduced the symptoms of the disease and the same organism was recovered from the diseased tissue. Biochemical studies showed that the intracellular proteins of the bacterial isolate contained appreciable catalase activity. Also higher levels of POX were observed in spongy pulp, indicating a defence response, against the bacterial infection. Air cavities observed in spongy fruits could be attributed to the expression of higher catalase activity by the organism to counteract the host defence response, as well as an increased respiration rate of the diseased fruits (Janave 2007). *Staphylococcus xylosus* has not been previously reported as a pathogen of mango. Occurrence of spongy tissue in mangoes is considered as an internal physiological disorder or ripening-associated disorder (Gupta et al. 1985; Katrodia 1988; Lima et al. 2001; Ravindra and Shivashankar 2004; Raymond et al. 1998a, b; Thomas et al. 1993; Vasanthaiah et al. 2006). However, the results presented here indicate that this disorder may not be a physiological or ripening-associated disorder, and provide evidence that *S. xylosus* infection could be an important factor for the initiation of spongy tissue in Alphonso mango

fruits. Further studies are necessary to develop control strategies against this organism in order to prevent the occurrence of spongy tissue in this economically important fruit crop of India.

Submission of nucleotide sequence to GenBank The nucleotide sequence of the *S. xylosus* strain has been deposited in the GenBank database under accession no. EU019195, gi 153946373, and the culture has been deposited in the Microbial-Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India, under the Accession number MTCC 7441.

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